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(57) Abstract

The present invention provides a method for protecting proteins against aerosolization-induced degradation and loss of protein activity. The aerosolized proteins are stabilized by adding a water soluble polar organic compound, such as polyethylene glycol, or a surfactant to the aqueous solution prior to aerosolization. The present invention further provides a stabilized aqueous protein solution containing a polar organic compound or surfactant and a system for aerosolizing the stabilized aqueous protein-containing solution.

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STABILIZATION OF AEROSOLIZED PROTEINS

Background

Aerosolization is an important method of drug delivery. An aerosolized drug is inhaled by the patient, bringing the drug into contact with the patients lungs. Because the airways in the lungs provide a large surface area for adsorption, aerosolization is useful not only for local delivery of drugs (*e.g.*, bronchodilators and other anti-asthma drugs), but also for systemic administration. In fact, aerosolization is believed to be a preferred route for the non invasive systemic administration of certain drugs, including proteins. In general, the size of the aerosolized particle is adjusted to control whether the particle reaches the small airways and alveoli of the lungs (necessary for systemic administration) or is delivered throughout the respiratory tract (localized delivery).

Aerosolized drugs are generated by dispersing either a dry particulate or an aqueous or non aqueous solution containing the drug into a gaseous medium. In the case of a dry particulate, the particles are suspended in propellants which evaporate after the suspension is released from a pressurized device into the air. Alternatively, the dry particulate can be aerosolized directly from a dry powder inhaler device. Where the drug is aerosolized from an aqueous solution, the drug solution is converted into a fine spray of minute water droplets.

Aerosolized delivery of a drug can be by means of a single metered dose or through continuous delivery.

One means for metered dose delivery of an aerosolized drug is a device referred to as a metered dose inhaler (MDI). When actuated, an MDI disperses a suspension of fine particles from a pressurized container. The patient inhales simultaneously upon actuating the inhaler, thus drawing the aerosolized drug into contact with the patient's lungs. Most typically, the drug delivered through an MDI is aerosolized from a dry particulate, but it is also possible to deliver a single metered dose of a drug aerosolized from an aqueous solution.

The use of an MDI requires a certain amount of coordination and technique that may not be reasonably expected from certain patients, such as the very young, the elderly and the infirm. In addition, there are only a limited number of drugs that can be readily formulated for use in an MDI. As a consequence, continuous delivery of an aerosolized drug is often preferred. The most common method for continuous delivery of an aerosolized drug is by a nebulizer which administers the drug to a patient who inhales the drug through normal breathing over an extended period of time. In order to provide the continuous flow necessary for administration over an extended period of time, an aqueous solution of the drug is continuously converted to a spray within the nebulizer, with only a small amount (approximately 1%) of the aqueous spray leaving the nebulizer directly for delivery to the patient at any given time. The aqueous spray that does not escape from the nebulizer impacts on the walls or baffles of the nebulizer and drains back to the fluid reservoir at the bottom of the nebulizer where it is again aerosolized into an aqueous spray until the reservoir is depleted or until drug administration is otherwise terminated.

Both metered dose and continuous flow delivery of aerosolized drugs, such as bronchodilators, corticosteroids, antibiotics, antihistamines and mucolytics, have been widely used for many years. More recently, advances in molecular biology have led to the development of a number of protein-based drugs, such as growth factors and cytokines, which have been suggested for aerosolized delivery, *e.g.*, as disclosed in European Patent Publication No. 0257956. As alluded to earlier, aerosolization is considered to be a preferred alternative delivery route for proteins that are impeded by oral delivery due to barriers such as instability toward proteolytic enzymes. However, aerosolized delivery of proteins introduces new challenges due to the delicate nature of these proteins as compared with more traditional drugs.

A potential problem with the aerosolized delivery of certain proteins is the delicate nature of the quaternary, and also secondary and tertiary, structure of the protein molecule which, if disrupted, leads to aggregation and degradation of the protein, resulting in loss of biological activity. The physical stresses inherent in aerosolization, such as the formation of a large area of air-water interface, destabilize the structure of many proteins. This problem is exacerbated in the case of continuous flow delivery, where approximately 99% of the drug in aqueous solution is refluxed, *i.e.*, aerosolized from aqueous solution more than once before it is delivered to the patient. This refluxing of the drug solution introduces additional physical stresses to the protein with each aerosolization cycle.

It is an object of the present invention to provide a method for preventing activity loss and degradation and/or aggregation of proteins aerosolized from an aqueous solution.

It is a further object of the present invention to provide a stabilized aqueous protein-containing solution that is resistant to degradation, aggregation and loss of protein activity upon aerosolization.

Summary of the Invention

The present invention provides a method for stabilizing proteins aerosolized from an aqueous solution. The aerosolized proteins are stabilized by adding a water soluble polar organic compound, such as polyethylene glycol, or surfactant to the aqueous solution prior to aerosolization. The present invention further provides a stabilized aqueous protein solution containing a polar organic compound or surfactant and a system for aerosolizing the stabilized aqueous protein-containing solution.

Brief Description of the Drawings

FIGURE 1 is a graph plotting the fraction of initial activity of LDH versus time of aerosolization.

FIGURE 2 is a non-denaturing electrophoretic gel showing G-CSF aerosolized from an aqueous solution containing PEG and an aqueous solution not containing PEG ("control").

FIGURE 3 is a graph plotting the percent degradation of G-CSF versus time of aerosolization both with 1% PEG and without PEG ("control").

FIGURE 4 is an SDS electrophoretic gel showing G-CSF aerosolized from an aqueous solution containing PEG and an aqueous solution not containing PEG ("control").

FIGURE 5 is a graph plotting the fraction of initial activity of LDH versus time of aerosolization for LDH aerosolized from an aqueous solution containing PEG and an aqueous solution not containing PEG ("control").

FIGURE 6 is a graph plotting the fraction of initial activity of LDH versus time of aerosolization for LDH aerosolized from an aqueous solution containing various concentrations of PEG and an aqueous solution not containing PEG ("control").

FIGURE 7A is graph showing the effect of different molecular weights of PEG, at a concentration of 0.001%, on stabilization of G-CSF during aerosolization.

FIGURE 7b is graph showing the effect of different molecular weights of PEG, at a concentration of 1%, on stabilization of G-CSF during aerosolization.

Detailed Description of the Invention

The present invention provides a method for protecting proteins against aerosolization-induced degradation, aggregation and/or loss of protein

activity. The present invention can be used in any situation where aerosolization of a protein is desired, including pulmonary delivery of an aerosolized protein-based drug to a patient. As noted, the present invention further provides a stabilized aqueous protein-containing solution that is resistant to degradation, aggregation and/or loss of protein activity upon aerosolization and a system for aerosolizing the stabilized aqueous protein-containing solution. In most cases, the system for aerosolizing the stabilized protein-containing solution will be used in the pulmonary administration of an aerosolized drug to a patient.

In accordance with the teachings of the present invention, aerosolized proteins are protected from activity loss, degradation and/or aggregation upon aerosolization by the addition of a polar organic compound or surfactant to the aqueous solution containing the protein.

In order to aid in the understanding of the present invention, the following terms, as used herein, have the definitions designated below.

The term Polar Organic Compound means an organic compound having a moderate non-polar moiety in an undefined region of the molecule (*i.e.*, the non-polar moiety is not localized). Polar organic compounds are capable of reducing the surface tension of water. Unlike surfactants, which tend to form micelles, polar organic compounds are completely miscible with water without the formation of micelles.

The term Surfactant means a compound having both a polar moiety and a strong non-polar moiety, each of which is contained in a separate localized region of the molecule, such that the surfactant can reduce interfacial tension between two immiscible phases by aligning at the interface and can form micelles. Because of the localization of the polar and non-polar moieties in surfactants, these compounds decrease the surface tension of water to a much greater extent than polar organic compounds.

The term Protein refers to any protein that is dispersed in the aqueous solution of the present invention. Ordinarily, the protein will be a protein of therapeutic utility that is intended for inhalation therapy. The protein may be chemically synthesized or purified from a natural source, but will typically be a recombinant form of a naturally occurring protein. The protein may also be chemically modified, usually by the covalent attachment of a chemical moiety to the protein molecule to enhance its therapeutic effect, such as extending the therapeutic effect of the protein.

As noted, aerosolization of proteins can result in a time-dependent loss of protein activity. Aerosolization adversely affects protein stability due to several factors, including the large amount of air-water interface created in the aerosolization process. The degree of loss of protein activity from aerosolization will vary from protein to protein depending on the structure and stability of the particular protein and the means used to achieve aerosolization. In the case of proteins continuously aerosolized by a jet nebulizer, shear forces from the air pressure of the jet disrupt the protein in addition

to the interaction of the protein with the air-water interface created in the aerosolization process. Drying may also cause denaturation of the protein as it becomes concentrated in the evaporating droplets. Thus the degree of activity loss from aerosolization using a jet nebulizer is related to both the air pressure of the jet and the starting volume of aqueous solution in the reservoir. In the case of proteins aerosolized by a sonic nebulizer, shear forces are not present in the same form, but ultrasonic waves can denature the protein in other ways (*e.g.*, heating).

Not all proteins suffer degradation, aggregation and/or and activity loss during aerosolization. For example, secretory leukoprotease inhibitor (SLPI) and α1-antitrypsin have been reported to be resistant to degradation upon aerosolization. McElvaney *et al.*, *J. Clin. Invest.*, *90*, 1296-1301 (1992) (SLPI); Hubbard *et al.*, *Ann. Int. Med.*, *111*(3), 206-212 (1989) (α1-antitrypsin). Some proteins may degrade partially or completely upon aerosolization, depending upon the susceptibility of the protein to the stress forces present during aerosolization. Most commonly, aerosolization will cause partial degradation of a protein. Partial degradation does not prevent efficacious use of the protein as a therapeutic, but does require the administration of a greater amount of the protein to achieve an efficacious level of drug than would be required if the protein were delivered entirely in its active form. There is also the possibility that the immunogenicity of aggregated protein will be different from the biologically active form, thus causing an immune response from the patient to whom the aerosolized drug is administered.

The exact mechanism by which proteins suffer activity loss and degradation and/or aggregation during aerosolization is unknown. Analysis of an aqueous solution containing the drug granulocyte colony stimulating factor (G-CSF) demonstrated that G-CSF itself decreases the surface tension of water even before aerosolization, indicating an orientation of this protein at the air-water interface. (At a concentration of 4 mg/ml, the surface tension of an aqueous solution of G-CSF was measured at 48, as compared to 72 for water.) Typically, adsorption of proteins at the air-water interface in an aqueous solution involves at least partial unfolding of the protein to expose the hydrophobic regions of the protein to the non-polar air phase. Analysis of an aqueous solution of G-CSF demonstrated homogeneity of the protein at this point (*i.e.*, prior to aerosolization), with the protein appearing as a single band on a non-denaturing electrophoretic gel and no evidence of aggregation being evident. This indicates that the surface adsorption of G-CSF prior to aerosolization is probably a reversible process. Upon aerosolization, however, G-CSF was observed to be irreversibly denatured, consequently suffering aggregation, chemical degradation, or both.

G-CSF is a potent growth factor of granulocyte lineage that enhances the number of white blood cells. Because of this activity, it has a beneficial clinical application in treating a number of diseases and conditions, including infectious disease. The current commercial route for administering G-CSF has been intravenous injection exclusively, but efficacy has been demonstrated in animals by means of pulmonary administration. (International Patent Publication No. WO 92/16192.) However, due to the demonstrated

aggregation, degradation and activity loss of the protein upon aerosolization, much of the aerosolized G-CSF can be expected to reach the patient in inactive form as covalent and non-covalent dimers and higher molecular weight aggregates and other degraded forms. Specifically, an attempt was made to nebulize 4 mg/ml solutions of G-CSF at acidic pH. Upon this treatment, the protein appeared to suffer damage in the form of both aggregation and degradation. Both of these types of damage increased with the length of nebulization.

A marked stabilization of aerosolized proteins against degradation was surprisingly observed when certain polar organic compounds or surfactants, as defined above, were included in the aqueous solution from which the proteins were aerosolized in accordance with the teachings of the present invention. This marked stability was demonstrated in connection with both the enzyme lactate dehydrogenase (LDH) and the aforementioned G-CSF. Preferred polar organic compounds found to have a protective effect on aerosolized proteins include polyethylene glycol (PEG) and methyl pentanediol (MPD). It is more preferred that the polar organic compound be PEG. It is most preferred that the polar organic compound be PEG 1000 (*i.e.*, PEG with an average molecular weight of 1000), although other molecular weight PEGs are also effective, although the lower molecular weight PEGs must be used at a higher concentration to achieve optimum protection of the aerosolized protein. A preferred surfactant is Tween 80.

Polyethylene glycol is not known for its ability to stabilize proteins. In fact, PEG is better known for its ability to destabilize proteins due to its

weak hydrophobicity. Arakawa and Timasheff, *Biochemistry, 24*(24), 6756-6762 (1985). This compound is also known to salt-out proteins due to its large excluded volume. *Ibid.* Functionally, PEG is strongly excluded from the surface of proteins, due to steric exclusion principles. This thermodynamically unfavorable interaction between PEG and proteins from whose surface PEG is excluded, results in decreased solubility of the proteins. As a consequence, PEG would be expected to destabilize proteins and decrease the solubility of proteins such as G-CSF, thus encouraging protein aggregation.

However, as noted, PEG has surprisingly been found to stabilize both LDH and G-CSF during nebulization from an aqueous solution. This may be due to the repeating nature of the amphiphilic units created by the non-localized polar and non-polar regions of the PEG molecule. In fact, PEG decreases the surface tension of water, although to a much lesser extent than common surfactants such as SDS and Tween. Thus, PEG is adsorbed at air-water interface, which may exclude proteins, such as G-CSF, from coming to the surface.

It is believed that the mechanism by which other polar organic compounds may protect proteins during aerosolization is also related to the weak capacity of these polar organic compounds to decrease surface tension. The protection afforded by PEG is concentration dependent, as has also been demonstrated for MPD. The protective effect afforded by other polar organic compounds is likewise expected to be concentration dependent, although the optimum concentration for achieving protection against degradation and activity loss will vary from one polar

organic compound to another and will also depend upon the protein being aerosolized. The optimum concentration for a polar organic compound in combination with a given protein will be readily ascertainable by one of ordinary skill in the art, following the teachings of the present invention. For example, it is preferred that the polar organic compound be employed at a concentration sufficient to reduce the surface tension of water to no greater than about 65 dynes/centimeter. In the case of MPD, it is more preferred to use the polar organic compound at a concentration capable of reducing the surface tension of water to no greater than about 48 dynes/centimeter.

The polar organic compounds contemplated for use in connection with the present invention are to be contrasted with certain surfactants, also known to decrease surface tension, that have been suggested for reducing or preventing surface induced aggregation of the protein caused by aerosolization of the protein from an aqueous solution. These surfactants include "conventional" surfactants such as polyoxyethylene fatty acid esters and alcohols, and polyoxyethylene sorbitan fatty acid esters, with an especially preferred surfactant being polyoxyethylene sorbitan monooleate. International Patent Publication No. WO 92/16192. Interestingly, these surfactants have been concluded as not being necessary for the aerosolization of G-CSF from an aqueous solution. *Ibid.*

Surfactants such as Tween may also be capable of lowering surface tension, in fact to a significantly greater extent than PEG or other polar organic compounds contemplated for use in the practice of the present invention. However, surfactants (particularly cationic

surfactants), unlike the polar organic compounds taught by the present invention, can form micelles and destabilize proteins. One reason for this is that, although cationic surfactants lower surface tension, they also bind strongly to the protein, particularly in micelle form, thus altering its stability. Moreover, the tendency of surfactants to cause foaming makes it somewhat difficult to utilize the surface tension reducing properties of these compounds in air-jet nebulization in many instances. Nevertheless, it may sometimes be preferred to use a surfactant to stabilize a protein against degradation. Where a surfactant is used for this purpose, it is preferred that the surfactant be employed at a concentration sufficient to reduce the surface tension of water to no greater than about 40 dynes/centimeter. The polar organic compounds contemplated by the present invention have the advantage of being able to lower surface tension without binding to and destabilizing the protein. Also, in the case of PEG, the random coil formation of PEG in aqueous solution and the large hydrodynamic volume that even small molecular weight PEGs occupy may add to their ability to prevent proteins from reaching the air interface generated during nebulization.

Contemplated for use in the practice of the present invention are a variety of proteins for which aerosolization from an aqueous solution would be desired. Most typically, aerosolization of proteins according to the present invention will be for pulmonary delivery of a therapeutically useful protein. Exemplary proteins contemplated are cytokines, including various hematopoietic factors such as the aforementioned G-CSF, SCF, EPO, GM-CSF, CSF-1, the interleukins such as IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10,

IL-11 and IL-12, IGFs, M-CSF, thymosin, TNF, or LIF. Other therapeutic proteins such as interferons (alpha-, beta-, gamma- or consensus interferons) and growth factors or hormones are also useful, such as human or other animal growth hormones (for example, bovine, porcine, or chicken growth hormone), ET-1, FGF, KGF, EGF, IGF, and PDGF. Protease inhibitors, such as metalloproteinase inhibitors are contemplated (such as TIMP-1, TIMP-2, or other proteinase inhibitors). Nerve growth factors are also contemplated, such as BDNF and NT3. Plasminogen activators, such as tPA, urokinase and streptokinase are also contemplated. Also contemplated are peptide portions of proteins having all or part of the primary structure of the parent protein and at least one of the biological properties of the parent protein. Analogs, such as substitution or deletion analogs, or those containing altered amino acids, such as peptidomimetics are also contemplated.

Contemplated for use in the practice of this invention are a wide range of mechanical devices designed for pulmonary delivery of therapeutic products aerosolized from aqueous solution, including but not limited to nebulizers and metered dose inhalers, which are familiar to those skilled in the art. It will be appreciated, however, that the greatest advantage of the present invention lies in its use in connection with continuous aerosolization of proteins, such as by a nebulizer, because of the increased degree of degradation ordinarily encountered with continuous aerosolization of proteins. Some specific examples of commercially available devices suitable for the practice of this invention are the Ultravent nebulizer, manufactured by Mallinckrodt, Inc., St. Louis, Missouri, the Acorn II nebulizer, manufactured by Marquest Medical

Products, Englewood, Colorado and the Collison 3-jet nebulizer, manufactured by BGI, Inc., Waltham, Massachusetts.

Formulations suitable for use with a nebulizer, either jet or ultrasonic, will typically comprise the protein to be aerosolized (or chemically modified protein) dissolved in water. Where the protein is G-CSF, the concentration should be about 0.1 to 25 mg of G-CSF per ml of solution. The formulation may also include a buffer or may simply be aqueous HCl. Examples of buffers which may be used are sodium acetate, citrate and glycine. Preferably, for G-CSF formulations, the buffer will have a composition and molarity suitable to adjust the solution to a pH in the range of 2.5 to 5.5. Generally, buffer molarities of from 1 mM to 50 mM are suitable for this purpose.

A means for aerosolizing the stabilized aqueous protein-containing solution of the present invention may be provided along with the aqueous solution to provide a system for generating an aerosolized protein. Although the system of the present invention may be useful in any situation where the aerosolization of proteins is desired, it is anticipated that this system will be most useful in the pulmonary administration of aerosolized protein-based drugs.

In this connection, the present invention contemplates the administration of therapeutic amounts of aerosolized protein sufficient to achieve the desired therapeutic effect. What constitutes a therapeutically effective amount of the protein or in a particular case will depend on a variety of factors which the knowledgeable practitioner will take into account,

including the desired therapeutic result, the severity of the condition or illness being treated, the physical condition of the subject, and so forth. In the case of G-CSF administration, a dosage regimen will be followed such that the normal blood neutrophil level for the individual undergoing treatment is restored, at least in cases of abnormally low or depressed blood neutrophil counts. For humans, the normal blood neutrophil level is about 5000 to 6000 neutrophils per microliter of blood. Neutrophil counts below 1000 in humans are generally regarded as indicative of severe neutropenia and as placing the subject at great risk to infection. Clinical studies with cancer patients suffering from chemotherapy-induced neutropenia have shown that subcutaneous injected doses of 3-5 μg G-CSF/kg every twenty-four hours are effective in elevating acutely deficient blood neutrophil levels above 1000.

As those skilled in the art will recognize, the operating conditions for delivery of a suitable inhalation dose will vary according to the type of mechanical device employed. For some aerosol delivery systems, such as nebulizers, the frequency of administration and operating period will be dictated chiefly by the amount of protein or other active composition per unit volume in the aerosol. In general, the higher the concentration of protein in the nebulizer solution the shorter the operating period. Some devices such as MDIs may produce higher aerosol concentrations than others and thus will be operated for shorter periods to give the desired result.

The following examples are provided to aid in the understanding of the present invention, the true scope of which is set forth in the appended claims. It

is understood that modifications can be made in the procedures set forth, without departing from the spirit of the invention.

Example 1

Aerosolization of Lactate Dehydrogenase

The influences of time, air pressure and nebulization volume were first examined with lactate dehydrogenase (LDH) solution as a model protein solution, because of ease of measuring the stability of this protein by using standard enzyme activity assay techniques to quantify enzyme activity as a measure of biologically active (*i.e.*, undegraded) protein.

Starting volumes of 10 ml of an aqueous solution containing LDH at a concentration of 25 µg/ml were nebulized using a Collison 3-jet nebulizer (BGI, Inc., Waltham, Massachusetts) and compressed air pressures of 10, 25 or 40 psig. Aliquots of 100 µl were removed from the reservoir fluid at t = 0, 2, 5, 5, and 10 minutes for each sample and assayed for enzyme activity.

Aerosolization of LDH under each of the conditions set forth above resulted in an irreversible, time dependent loss of enzymatic activity in the reservoir solution, as demonstrated by electrophoretic/densitometric analysis. On average, approximately 60% of the starting enzyme activity was lost after 60 minutes of nebulization at 40 psig, as shown in Figure 1. The loss of activity was reduced by progressively lowering the applied air pressure, but not substantially (see Figure 1).

Example 2

Aerosolization of G-CSF

Solutions of G-CSF were nebulized according to the same protocol set forth in Example 1 for LDH, with samples of the aerosolized protein being removed for analysis by the chromatographic and electrophoretic/densitometric procedures described below.

Analysis of SE HPLC of samples of G-CSF solution removed from the reservoir of the nebulizer during aerosolization demonstrated the progressive formation of an aggregate, as indicated by elution of this aggregated form of the protein from the HPLC column prior the monomeric peak. The aggregated form of the protein was observed within 15 seconds of nebulization and the relative fraction of aggregated protein appeared to reach a plateau after about 5 minutes of nebulization. Typically, this was 25-30% of the integrated peak areas. This aggregate appeared to be non-covalent since aggregate bands did not appear on SDS-PAGE gels. Instead, only a monomeric band was observed.

A homogeneous 6% non-denaturing (*i.e.*, in the absence of SDS) Novex gel was used to evaluate the aerosolized G-CSF. An electrode buffer contained 25 mM Tris and 20 mM glycine. The sample for the gel contained the same concentrations of 25 mM Tris and 20 mM glycine, along with 2.5% glycerol, 2.5% sucrose, and 0.025% β-bromophenol blue. A constant 50 V was applied to the gel, with a total running time of approximately 7 hours. Approximately 30 mg of G-CSF was

loaded onto the gel. Each gel was then stained with Coomassie blue. After destaining, if necessary, the gels were subjected to densitometric analysis using an LKB Ultrascan® XL laser densitometer.

Analysis of the aerosolized protein solution using gel electrophoresis under native conditions revealed formation of a new band that migrated with a slightly higher mobility than the intact molecule. This new band, representing degraded protein was observable after as little as one minute of treatment, as shown in Figure 2 ("control" side of gel). Figure 2 further shows that this new band increased in intensity and appeared to saturate with increasing time of nebulization. The percentage of the new band is plotted in Figure 3, showing that conversion occurs rapidly with a half time of 20 minutes and saturates at the 40% level at around 5 minutes. Similar degradation, with slightly varying degrees, was observed between pH 3 and 4 in aqueous HCl or glycine buffer. The roughly similar mobility of the degraded band to the intact protein suggests that the protein is still at least partially folded. A fully unfolded molecule would migrate much more slowly in the native gel. SDS-PAGE analysis of these samples yielded virtually a single band, as shown in Figure 4 ("control" side), suggesting that the altered mobility is due to a change in the charged state, such as more negative charges, rather than peptide cleavages.

Example 3

Effects of Polar Organic Compounds on Protein Stability

Based on the observed instability of LDH and G-CSF aerosolized from aqueous solution in Examples 1 and 2, respectively, different concentrations of PEG and MPD were added to these protein-containing aqueous solutions in an attempt to minimize the observed degradation. Specifically, PEG 1000 and 2-methyl-2,4-pentanediol were added to different aqueous solutions containing either LDH or G-CSF. Concentrations of 0.1%, 1% and 10% (w/v) PEG 1000 and 1% and 5% and 10% (w/v) MPD were used to determine the optimum concentrations of the polar organic compounds for the stabilization of the two proteins being studied. The resulting solutions were aerosolized, with the aerosolized proteins being analyzed by gel electorophoresis and densitometry, as set forth in the previous examples.

An LDH solution including 1% PEG 1000 resulted in almost complete retention of the initial activity of the protein over a 60 minute period of nebulization at 40 psig, as shown in Figure 5. The protective effect of PEG was also found to be a function of the PEG concentration, as shown in Figure 6. Protein formulated in 1% 2-methyl-2,4-pentanediol (MPD) retained substantially all of its enzymatic activity over the nebulization period.

Similar results were obtained for aqueous solutions containing G-CSF and varying concentrations of the two polar organic compounds. Protein-containing solutions to which the one of the polar organic compounds had been added showed a significant decrease

in both the amount of aggregate and the intensity of the "degraded band" observed with non-denaturing PAGE as compared to G-CSF nebulized without PEG. The formation of the degraded protein occurred much more slowly, and the degradation appeared to saturate at less than 10% in the presence of PEG, versus 40% for the control sample (see Figure 3). Analysis of the degraded protein using SE HPLC also demonstrated concentration dependence for stabilization of G-CSF with both PEG and MPD.

Example 4

Comparative Measurements for G-CSF Aerosolized from Different Aqueous Solutions

Surface tension values were evaluated for aqueous solutions containing different molecular weights and concentrations of PEG and MPD. In addition, sucrose, which has the opposite effect on the surface tension of water, was also examined in the context of protein stabilization. Table I reports the extent of G-CSF degradation observed following 10 minutes of nebulization as it corresponds to the surface tension value in water of the these compounds.

The surface tension of aqueous solutions was measured using the ring method of Denuoy (Leukenheimer and Wantke, *Colloid and Polymer Sci., 259,* 354 (1981) using a Krüss K10T surface tensiometer (Krüss Company, Hamburg, Germany). All measurements were made at 20°C. For all samples, at least 30 minutes were allowed prior to measurement for equilibration of the surface concentrations of the various components. All values were adjusted for density as described by Harkins and Jordan, *J. Amer. Chem. Soc., 52,* 1772 (1930). The

surface tension of pure water (Super Q®, Millipore) was measured prior to and after all sets of measurements to ensure accuracy. In addition, experiments were performed to ensure that potential protein deposition onto the tensiometer ring did not affect the measured values. The results are set forth in Table I.

Table I

Aqueous Solution	Degradation (%) at 10 Minutes	Surface Tension (dynes/cm)
1 mM HCl (control)	39	72
1% PEG 1000	5-8	65*
1% MPD	24-31	58
5% MPD	2-4	48
1% sucrose	43	72
0.5 M sucrose	44	75

*Cooper *et al.*, *J. Polymer Sci.*, *3*, 345-349 (1948)

This analysis demonstrates that both PEG and MPD exhibit a protective effect on the protein G-CSF during aerosolization, although the amount of the polar organic compound required to achieve a high level (*i.e.*, greater than 90% retention of activity) is higher for MPD (5%) than for PEG (1%).

Example 5

Comparative Effects of Certain Polar Organic Compounds and Surfactants on Protein Stability

Further experiments were conducted to examine the comparative effect of various polar organic compounds and surfactants on protein stability during nebulization. Specifically, polyoxyethylene sorbitan

mono-oleate (Tween 80), sucrose and methylpentanediol (MPD) (all from Sigma Chemical Co., St. Louis, Missouri) were tested for their capacity to stabilize G-CSF and LDH during nebulization. In addition, different molecular weights of PEG were analyzed to determine the difference in protective effect exhibited by the different molecular weights of these compounds. Specifically, PEG 8000 (average molecular weight of 8000) and PEG 400 (average molecular weight of 400) were studied in addition to the PEG 1000 utilized in the previous examples (all from Union Carbide.) The polar organic compounds and/or surfactants were added prior to nebulization, which was then performed according to the protocol described in Example 1, above.

Gel electrophoresis in the absence and presence of sodium dodecylsulfate was carried out as previously described in Example 2. Gel filtration chromatography was carried out using a Superose® 12 FPLC column (1 x 30 cm, Pharmacia, Uppsala, Sweden) and 0.1 M sodium phosphate, pH 6.9 as an eluent. The flow rate was set at 0.5 ml/minute, and elution was monitored with 280 nm absorbance. Surface tension measurements were determined according to the ring method as described in Example 4, above.

A. Effect of PEG Molecular Weight

The effect of PEG molecular weight on G-CSF stabilization during aerosolization was examined using molecular weights of 400, 1000 and 8000 daltons each at concentrations of 0.001% and 1.0% (w/v). The results are shown in Figures 7A and 7B, respectively. At a PEG concentration of 0.001% (w/v), there was essentially no correlation between protein stabilization and the PEG

molecular weight (Figure 7A). In contrast, a strong molecular weight dependence was observed at a PEG concentration of 1% (w/v), as shown in Figure 7B, with a definite trend toward greater stabilization being observed with increasing molecular weight of PEG.

B. Effect of MPD

MPD was tested for its ability to protect LDH and G-CSF during aerosolization. Like PEG, MPD is a protein stabilizer with weak surfactant activity. When LDH was formulated in 1% (w/v) MPD, the protein retained nearly 100% of the initial activity over the nebulization period relative to approximately only 33% retention in the absence of any additives. The stabilization effect of MPD against G-CSF degradation upon 10 minutes of nebulization is shown in Table II.

Table II

MPD Concentration (w/v)	Degradation (%) at 10 minutes
0%	39
0.01%	33
0.1%	35
1%	28
2.5%	15
5%	3
10%	0

The time course of G-CSF degradation was slower in the presence of MPD than the control experiment. Like PEG, stabilization of G-CSF by MPD was strongly concentration-dependent. In the case of MPD, this particular polar organic compound demonstrated a

minimal amount of stabilization below 0.1%. A substantial stabilization, although less than that achieved with equivalent concentrations of the higher molecular weight PEG, was observed with 1% MPD. A somewhat greater protection than obtained with 1% PEG 1000 was attained by 5% MPD, while 10% MPD appeared to confer virtually complete protection.

C. Effect of Sucrose

The effects of 2% (w/v, 0.058 M) or 20% (w/v, 0.58 M) sucrose on retention of LDH activity during nebulization were measured. The results showed that sucrose did not provide any significant protection against degradation, and, in fact, had a tendency to further destabilize LDH activity. The effects of 1% (w/v) and 0.5 M (17%) sucrose on G-CSF stabilization were also determined after 10 minutes of nebulization, with the results being nearly identical to those obtained for LDH. Thus, as earlier noted, the addition of sucrose did not have a protective effect against degradation during nebulization of G-CSF. In fact, sucrose demonstrated a potentially destabilizing effect.

D. Effect of Tween 80

Although excessive foaming of Tween 80 made nebulization difficult, the effect of this surfactant on G-CSF degradation during nebulization was examined at various different surfactant concentrations. The results are summarized in Table III, below.

Table III

Tween 80 (w/v)	(%) Degradation at 10 Minutes	Surface Tension
0%	40	72
0.0001%	39	51
0.001%	39	49
0.005%	36	
0.0075%	20	
0.01%	8	40
0.05%	0	
0.1%	0	38
1%	0	38

It is apparent from these results that Tween 80, like the polar organic compounds PEG and MPD, stabilizes G-CSF against degradation in a strongly concentration-dependent manner. Essentially no stabilizing effect was observed below a surfactant concentration of 0.001% (w/v), although substantial stabilization was observed at around 0.01% (w/v). Virtually complete stabilization was observed at surfactant concentrations above 0.05% (w/v). A similar stabilization pattern was observed with respect to LDH activity in the presence of Tween 80.

E. Surface Tension

Table IV summarizes the results of surface tension measurements of aqueous solutions of G-CSF and of the various polar organic compounds and/or surfactants examined in this and preceding example.

Table IV

Aqueous Solution	Degradation (%) at 10 Minutes	Surface Tension (dynes/cm)
1 mM HCl (control)	39	72
1% PEG 1000	5-8	65*
1% MPD	24-31	58
5% MPD	2-4	48
1% sucrose	43	72
0.5 M sucrose	44	75
0.0001% Tween 80	39	51
0.001% Tween 80	39	49
0.01% Tween 80	8	40
0.1% Tween 80	0	38
1% Tween 80	0	38

*Cooper *et al., J. Polymer Sci., 3,* 345-349 (1948)

It is apparent from the data in Table IV that both PEG and MPD, which decrease the surface tension of water, greatly reduce the amount of degradation observed in the G-CSF protein when used as additives during nebulization. On the other hand, sucrose, which slightly increases the surface tension of water, also slightly increased degradation of the protein. Tween 80 was shown to have a marked effect on the stability of G-CSF during nebulization, as shown in Table IV. This is typical of surfactants, but, as earlier noted, the tendency of surfactants to foam makes them less compatible than polar organic compounds for use with air-jet nebulization in many instances.

What is claimed is:

1. A method for preventing activity loss and degradation of a protein aerosolized from an aqueous solution containing said protein comprising adding to said solution a polar organic compound at a concentration which reduces the surface tension of water to no greater than about 65 dynes/centimeter.

2. The method of claim 1 wherein said polar organic compound is selected from the group consisting of polyethylene glycol and methyl pentanediol

3. The method of claim 2 wherein said polar organic compound is polyethylene glycol and said protein is granulocyte colony stimulating factor.

4. The method of claim 3 wherein said polyethylene glycol is PEG 1000 and is present at a concentration of at least about 1%.

5. The method of claim 2 wherein said polar organic compound is methyl pentanediol and reduces the surface tension of water to no greater than about 48 dynes/centimeter.

6. The method of claim 5 wherein said methyl pentanediol is 2-methyl-2,4-pentanediol, said protein is granulocyte colony stimulating factor and said 2-methyl-2,4-pentanediol is present at a concentration of at least about 5%.

7. The method of claim 1 wherein said protein is aerosolized by air-jet nebulization.

8. A method for preventing activity loss and degradation of a protein aerosolized from an aqueous solution containing said protein comprising adding to said solution a surfactant at a concentration which reduces the surface tension of water to no greater than about 40 dynes/centimeter.

9. The method of claim 11 wherein said surfactant is Tween 80, said protein is granulocyte colony stimulating factor and said Tween 80 is present at a concentration of at least about 0.01%.

10. An aqueous solution comprising a protein dispersed in water and a polar organic compound at a concentration which reduces the surface tension of water to no greater than about 65 dynes/centimeter.

11. The aqueous solution of claim 10 wherein said polar organic compound is selected from the group consisting of polyethylene glycol and methyl pentanediol

12. The aqueous solution of claim 11 wherein said polar organic compound is polyethylene glycol and said protein is granulocyte colony stimulating factor.

13. The aqueous solution of claim 12 wherein said polyethylene glycol is PEG 1000 and is present at a concentration of at least about 1%.

14. The aqueous solution of claim 11 wherein said polar organic compound is methyl pentanediol and reduces the surface tension of water to no greater than about 48 dynes/centimeter.

15. The aqueous solution of claim 14 wherein said methyl pentanediol is 2-methyl-2,4-pentanediol, said protein is granulocyte colony stimulating factor and said 2-methyl-2,4-pentanediol is present at a concentration of at least about 5%.

16. An aqueous solution comprising a protein dispersed in water and a surfactant at a concentration which reduces the surface tension of water to no greater than about 40 dynes/centimeter.

17. The aqueous solution of claim 16 wherein said surfactant is Tween 80, said protein is granulocyte colony stimulating factor and said Tween 80 is present at a concentration of at least about 0.01%.

18. A system for administering an aerosolized protein to a patient comprising the aqueous solution of claim 10 and a means for aerosolizing said aqueous solution.

19. The system of claim 18 wherein said aqueous solution comprises a protein dispersed in water and polyethylene glycol.

20. The system of claim 15 wherein said protein is granulocyte colony stimulating factor, said polyethylene glycol is PEG 1000 and said PEG 1000 is present at a concentration of at least about 1%.

21. The system of claim 18 wherein said aqueous solution comprises a protein dispersed in water and methyl pentanediol.

22. The system of claim 15 wherein said methyl pentanediol is 2-methyl-2,4-pentanediol, said protein is granulocyte colony stimulating factor and said 2-methyl-2,4-pentanediol is present at a concentration of at least about 5%.

FIG. 1

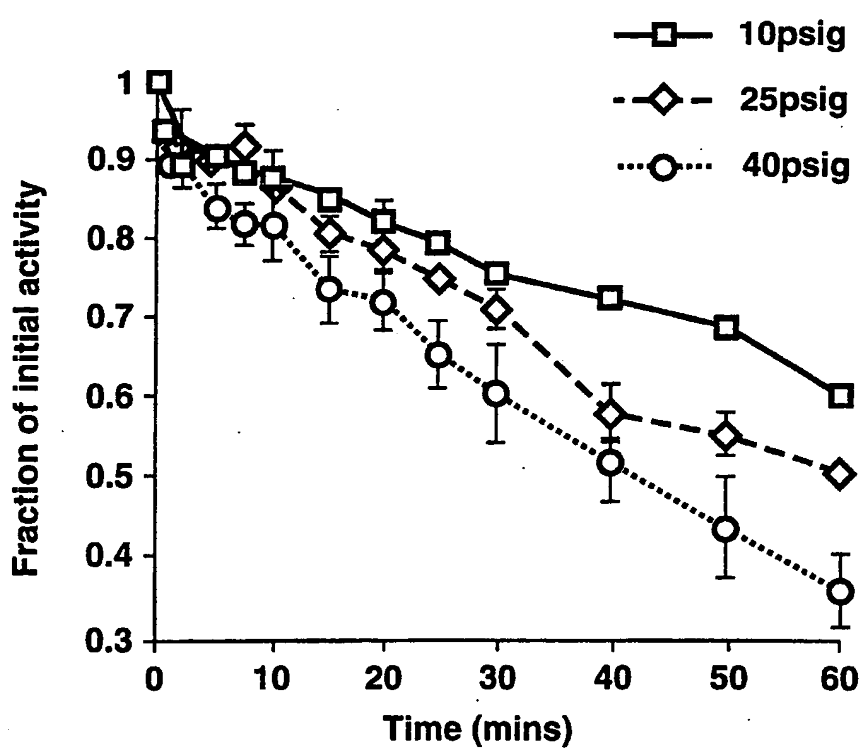

10psig
25psig
40psig
Fraction of initial activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0
10
20
30
40
50
60
Time (mins)

FIG. 2

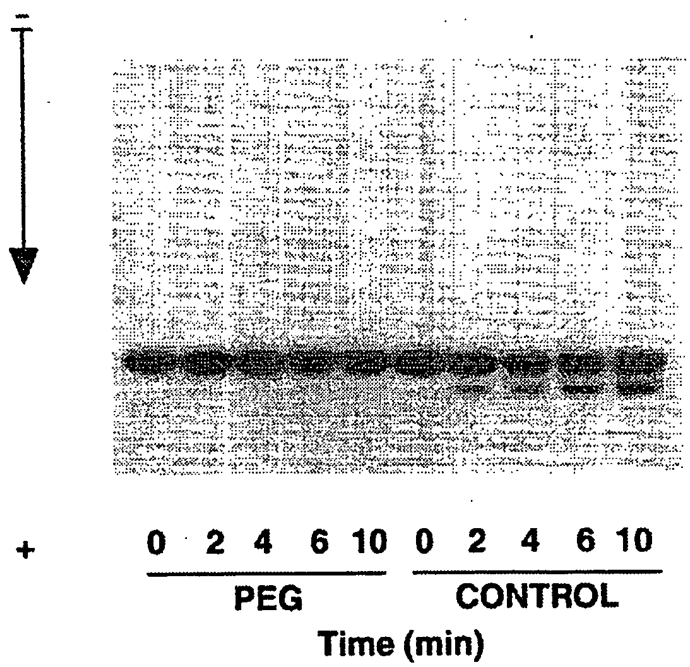


FIG. 3

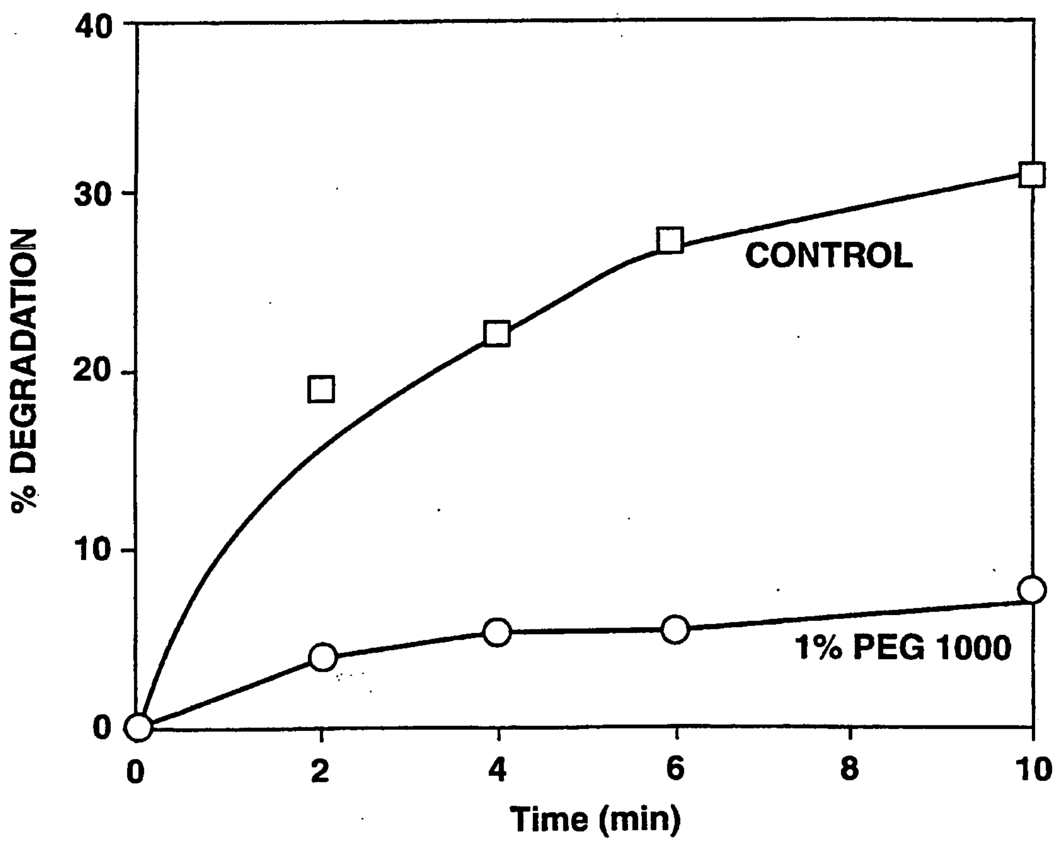


FIG. 4

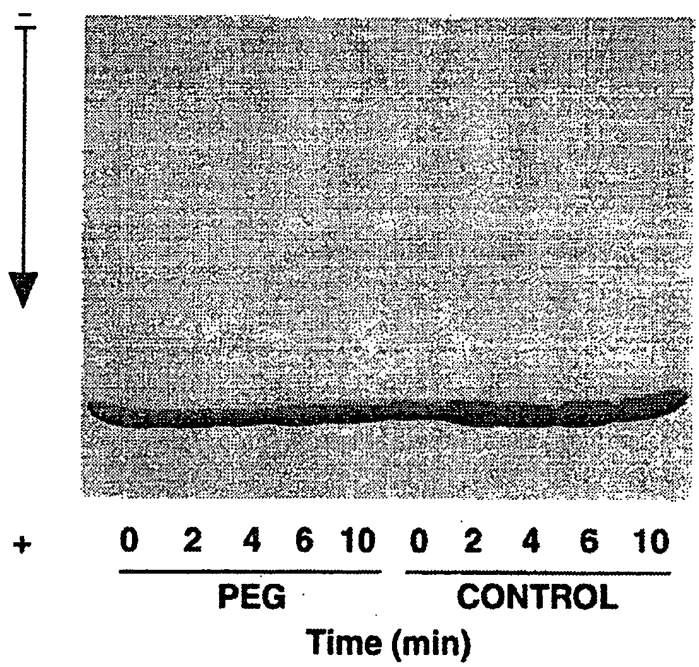


FIG. 5

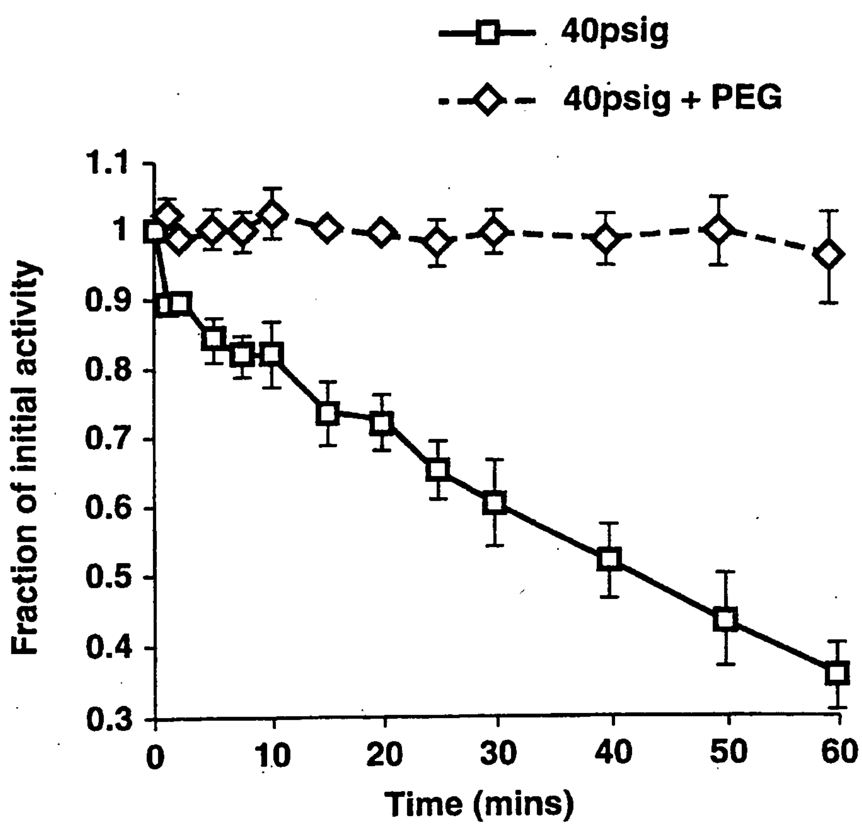

40psig
40psig + PEG
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
Fraction of initial activity
0
10
20
30
40
50
60
Time (mins)

FIG. 6

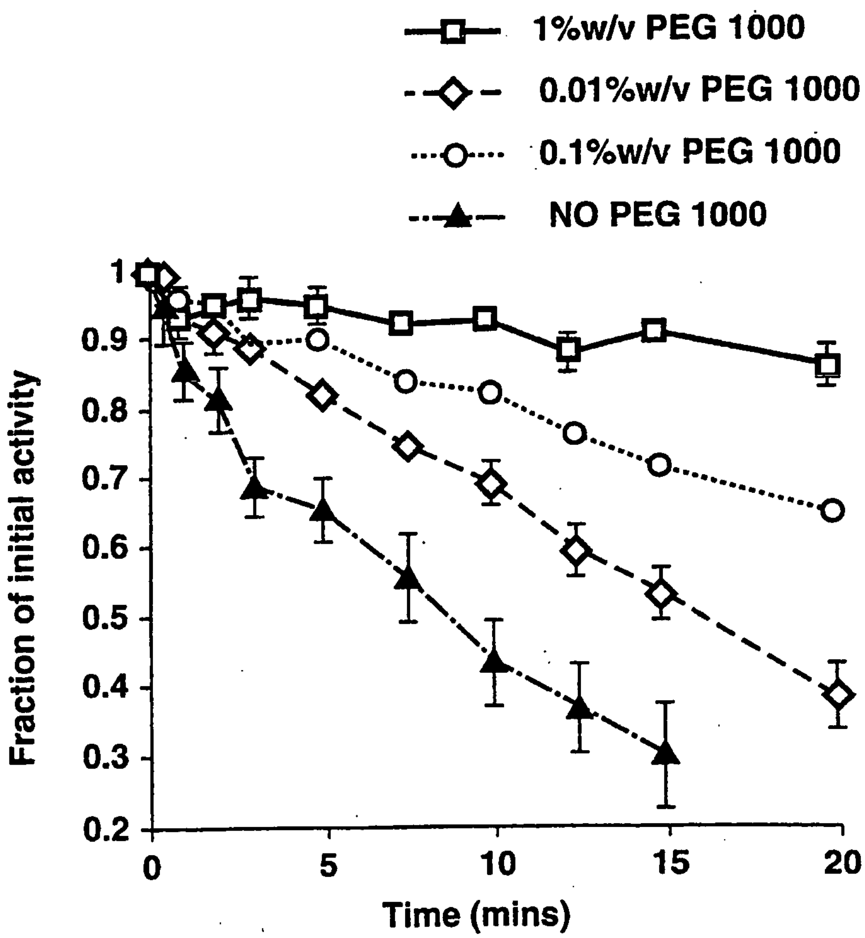

1%w/v PEG 1000
0.01%w/v PEG 1000
0.1%w/v PEG 1000
NO PEG 1000
Fraction of initial activity
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0
5
10
15
20
Time (mins)

FIG. 7A

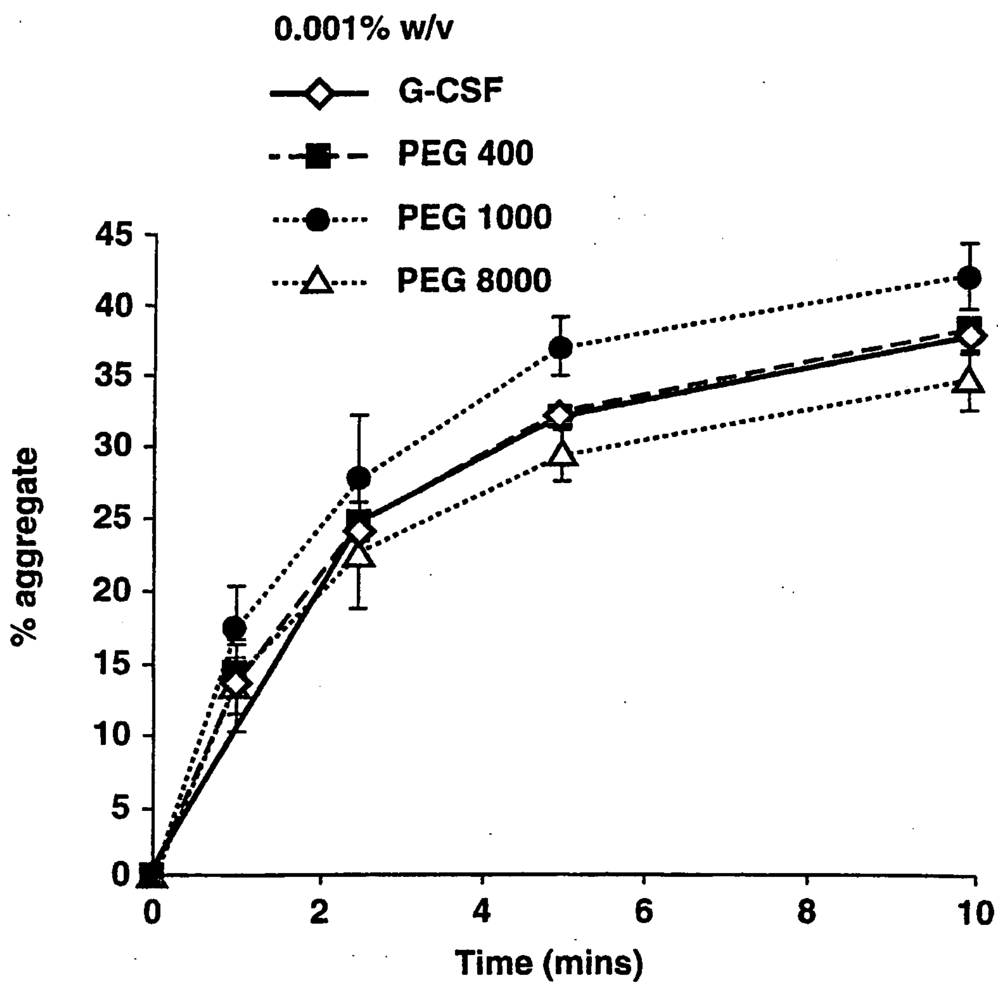
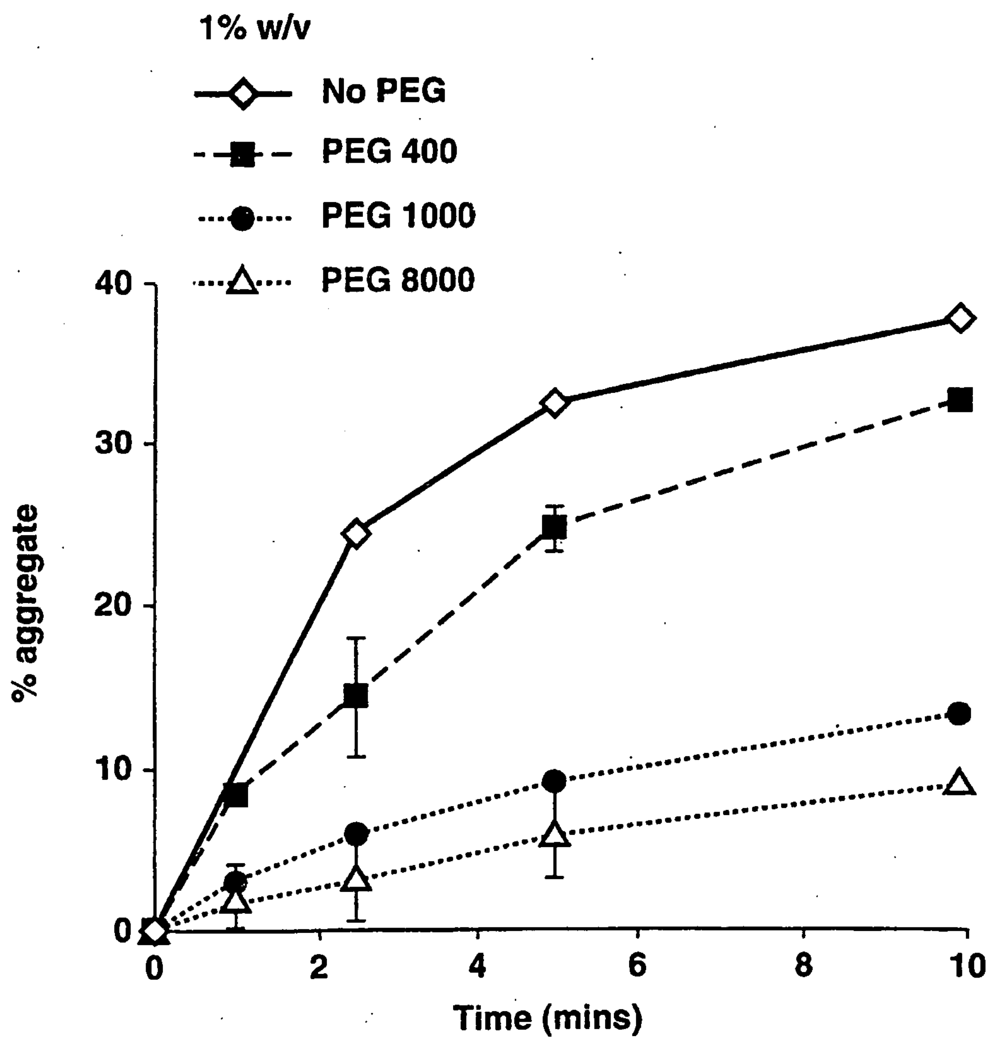

0.001% w/v
G-CSF
PEG 400
PEG 1000
PEG 8000
% aggregate
Time (mins)
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10

FIG. 7B


1% w/v
No PEG
PEG 400
PEG 1000
PEG 8000
40
30
20
10
0
% aggregate
0
2
4
6
8
10
Time (mins)

INTERNATIONAL SEARCH REPORT

Interr ıal Application No
PCT/US 94/08136

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K9/00 C12N9/96 A61M15/00 C07K14/53

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N A61M C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 510 731 (ABBOTT LABORATORIES) 28 October 1992 see the whole document ---	1-7, 10-22
X	WO,A,91 04011 (RIKER LBORATORIES) 4 April 1991 see page 3, line 1 - line 7 ---	1-7, 10-22
X P,X	WO,A,93 03708 (ZHYRNOV) 4 March 1993 & EP,A,0 563 389 (ZHYRNOV) 6 October 1993 see page 4, line 10 - line 20 --- -/--	8,9 8,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
29 November 1994	14 -12- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Masturzo, P

International Application No
PCT/US 94/08136

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	INTERNATIONAL JOURNAL OF PHARMACEUTICS, vol.109, no.1, 1994, AMSTERDAM, NL pages 17 - 26 R W NIVEN ET AL 'Protein nebulization: I. Stability of lactate dehydrogenase and recombinant granulocyte-colony stimulating factor to air-jet nebulization' -----	1-22

2

INTERNATIONAL SEARCH REPORT	In: .iational application No. PCT/US94/08136

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See annex.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Claim 9 has been read as being dependent on claim 8 and not on claim 11 ; there are two claims with the number 19, therefore the second has been considered to bring the number 22.
A polar organic compound (claim 1) is no clear definition ; this does not allow to perform a complete search ; therefore the search was limited to the surfactents and every other compound specifically mentioned in the application.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 94/08136

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0510731	28-10-92	US-A-	4897256	30-01-90
		US-A-	4851211	25-07-89
		CA-A-	1300009	05-05-92
		DE-A-	3785570	27-05-93
		DE-T-	3785570	18-11-93
		EP-A,B	0275404	27-07-88
		JP-A-	63211237	02-09-88
WO-A-9104011	04-04-91	AU-B-	648994	12-05-94
		AU-A-	6409790	18-04-91
		EP-A-	0493437	08-07-92
		JP-T-	5500664	12-02-93
		US-A-	5348730	20-09-94
WO-A-9303708	04-03-93	AU-A-	2517792	16-03-93
		EP-A-	0563389	06-10-93
EP-A-0563389	06-10-93	AU-A-	2517792	16-03-93
		WO-A-	9303708	04-03-93

Form PCT/ISA/210 (patent family annex) (July 1992)